Synthesis of Flavonoid *O*-Pentosides by *Escherichia coli* through Engineering of Nucleotide Sugar Pathways and Glycosyltransferase

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Plants produce two flavonoid *O*-pentoses, flavonoid *O*-xyloside and flavonoid *O*-arabinoside. However, analyzing their biological properties is difficult because flavonoids are not naturally produced in sufficient quantities. In this study, *Escherichia coli* was used to synthesize the plant-specific flavonoid *O*-pentosides quercetin 3-*O*-xyloside and quercetin 3-*O*-arabinoside. Two strategies were used. First, *E. coli* was engineered to express components of the biosynthetic pathways for UDP-xylose and UDP-arabinose. For UDP-xylose biosynthesis, two genes, *UXS* (UDP-xylose synthase) from *Arabidopsis thaliana* and *ugd* (UDP-glucose dehydrogenase) from *E. coli*, were overexpressed. In addition, the gene encoding ArnA (UDP-L-Ara4N formyltransferase/UDP-GlcA C-4″-decarboxylase), which competes with UXS for UDP-glucuronic acid, was deleted. For UDP-arabinose biosynthesis, *UXE* (UDP-xylose epimerase) was overexpressed. Next, we engineered UDP-dependent glycosyltransferases (UGTs) to ensure specificity for UDP-xylose and UDP-arabinose. The *E. coli* strains thus obtained synthesized approximately 160 mg/liter of quercetin 3-*O*-xyloside and quercetin 3-*O*-arabinoside.

Flavonoids are one of the largest groups of plant secondary metabolites (1), and approximately 10,000 different flavonoids are known (2). They are synthesized by the phenylpropanoid pathways (1). Most flavonoids are glycosylated at the hydroxyl groups during the last stage of biosynthesis (3). In plants, several sugars can be attached to flavonoids through ester linkages. Glucose is the sugar most commonly linked to flavonoids; other sugars linked to flavonoids include arabinose, galactose, glucuronic acid, rhamnose, and xylose (4).

Biosynthesis of flavonoid *O*-glycosides is mediated by nucleotide diphosphate-dependent glycosyltransferases (4). UDP-dependent glycosyltransferases (UGTs), which use UDP-sugars as sugar donors, are predominant among these; however, thymine derivatives are also used (5). Most UGTs display sugar acceptor specificity as well as sugar donor specificity (6). The N terminus of the UGTs is involved in the recognition of sugar acceptors, whereas the C terminus is involved in the recognition of sugar donors (6). The C terminus of plant UGTs contains a highly conserved motif (plant secondary product GT consensus sequence [PSPG]) that is critical for UDP-sugar recognition (3, 47).

Nucleotide sugars serve as the sugar donors in the biosynthesis of flavonoid glycones. In plants, nucleotide sugar biosynthesis has been well studied due to its importance in cell wall biosynthesis (8). Further, in plants, UDP-glucose is the precursor of other UDP-sugars, namely, UDP-galactose, UDP-glucuronic acid, and UDP-rhamnose, while GDP-mannose is the precursor of GDP-glucose and GDP-galactose (8–10). UDP-glucuronic acid serves as a precursor for UDP-galacturonic acid, UDP-xylose, UDP-adipose, and UDP-arabinose (10). Most of the genes involved in plant nucleotide sugar biosynthesis have been characterized (9). Plant-derived nucleotide biosynthetic genes are useful for engineering nucleotide biosynthesis pathways into heterologous systems.

Biological synthesis of phytochemicals using *Escherichia coli* has been widely studied (11–13). Biological synthesis provides both regioselectivity and stereoselectivity, which are difficult to achieve using chemical synthesis. Flavonoid glycosides have also been synthesized using *E. coli* expressing plant UGTs (14, 15).

Because most plant UGTs exhibit UDP-glucose specificity and *E. coli* provides UDP-glucose, most biotransformation products obtained using an *E. coli* system are flavonoid *O*-glucosides (16). However, other flavonoid *O*-glycosides, such as flavonoid *O*-rhamnoside and flavonoid *O*-xyloside, have been synthesized using *E. coli* by expressing plant UGTs along with the corresponding nucleotide biosynthesis genes (5, 17–19). The biological activities of a variety of flavonoid *O*-glycosides have been elucidated (20–23). Apart from flavonoid *O*-glucoside, other plant flavonoid *O*-glycosides such as flavonoid *O*-galactoside, flavonoid *O*-arabinoside, flavonoid *O*-glucuronic acid, flavonoid *O*-xyloside, and flavonoid *O*-rhamnoside can be isolated from plants only in small amounts with multiple purification steps. This makes it difficult to obtain these compounds in sufficient quantities to study their biological activities. Although flavonoid glycosides have been successfully synthesized chemically (24, 25), the utility of this approach is limited by its low regioselectivity and the involvement of multiple reaction steps. *In vitro* enzymatic synthesis is also impractical due to the high cost of nucleotide sugars (26). Therefore, production in a live host such as *E. coli* could offer advantages over chemical or enzymatic synthesis.

To synthesize plant flavonoid glycosides in *E. coli*, it is necessary to engineer the cells to express the components of the nucleotide biosynthesis pathway. Most bacteria, including *E. coli*, produce polysaccharides, and polysaccharide biosynthesis in *E. coli* is well understood (27). Similar to the case with plant cell wall biosynthesis, the building blocks for bacterial polysaccharide biosynthesis are also nucleotide sugars (28). These nucleotide sugars can be as sugar donors for various UGTs and, following introduction

TABLE 1 PCR primers used in this study

Primer	Sequence[a]	Accession number, designation, or code for source
AmUGT10 forward	ATGAATTCGATGGAGGACACTATCGTTCTC	GenBank accession no. AB362988
AmUGT10 reverse	CATGCGGCCGCTTAAGAAACCACCATATCA	GenBank accession no. AB362988
AtUXS forward	ATGGAATTCGATGGCAGCTACAAGTGAGAA	At5g59290
AtUXS reverse	ATGCGGCCGCTTAGTTTCTTGGGACGTTAA	At5g59290
Ecugd forward	AACATATGAAAATCACCATTTCCGG	GeneID 946571
Ecugd reverse	AACTCGAGTTAGTCGCTGCCAAAGAGA	GeneID 946571
Atugd forward	AACATATGGTGAAGATATGTTGTAT	At3G29360
Atugd reverse	AAGGTACCTTAGGCAACGGCAGGCATGT	At3G29360
Gmugd forward	AACATATGGTGAAGATTTGCTGCAT	GenBank accession no. U53418
Gmugd reverse	AACTCGAGTTATGCCACAGCAGGCATGT	GenBank accession no. U53418

[a] Restriction enzyme sites are underlined.

of exogenous genes into the cells, can serve as substrates in the production of other nucleotide sugars. Therefore, nucleotide sugars that are not normally found in *E. coli* can be biosynthesized by engineering the cells to express both bacterial and plant nucleotide sugar biosynthesis pathways.

Quercetin 3-*O*-xyloside and quercetin 3-*O*-arabinoside are mainly found in apple and mango (29, 30). Quercetin 3-*O*-xyloside binds dihydrofolate reductase (DHFR), which is a key enzyme for the biosynthesis of DNA, RNA, and amino acids and has potential as an anticancer drug (31, 32). It also shows promise against complications associated with diabetes by inhibiting lens aldol reductase (33). Quercetin 3-*O*-arabinoside inhibits accumulation of lipids (34, 35). Therefore, quercetin 3-*O*-pentosides have potential uses in medicinal foods or as drug candidates. Additional biological activities of these compounds could be explored if larger quantities of these compounds could be obtained. Therefore, in this study, we engineered *E. coli* to synthesize quercetin 3-*O*-xyloside and quercetin 3-*O*-arabinoside. Here, we report the biosynthesis of these compounds using a strain of *E. coli* containing mutations in its nucleotide biosynthesis pathway and harboring UGT and plant nucleotide synthesis genes.

MATERIALS AND METHODS

Nucleic acid manipulations. UDP-xylose synthase (*AtUXS*) from *Arabidopsis thaliana* (At5g59290 [36]) was cloned by reverse transcription-PCR (RT-PCR). Total RNA was isolated from 2-week-old *A. thaliana* plants using a plant total RNA isolation kit (Qiagen, Hilden, Germany), and cDNA was synthesized using 2 μg of total RNA, oligo(dT) primer, and Omniscript reverse transcriptase (Qiagen). PCR was carried out using hot start *Taq* DNA polymerase (Qiagen) under the following conditions: 40 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and amplification at 72°C for 1 min. The PCR primers used in this study are listed in Table 1. The gene for UDP-xylose epimerase (*OsUXE*) from *Oryza sativa* and *AtUGT78D3* were previously cloned in our laboratory (37, 38).

UDP-glucose dehydrogenase (*ugd*; GeneID 946571) was amplified using PCR with *E. coli* genomic DNA as a template. The PCR product was sequenced and subcloned into the NdeI/XhoI sites of pETDuet1 (Novagen, Darmstadt, Germany). *ugd* genes from *A. thaliana* (Gene ID At3G29360) and soybean (GenBank accession number U53418) were cloned using RT-PCR. The resulting PCR products were subcloned into the NdeI/KpnI (*Atugd*) or NdeI/XhoI (*Gmugd*) sites of pETDuet-1.

ArnA was deleted from *E. coli* BL21(DE3) the using the Quick and Easy Conditional Knockout kit (Gene Bridges, Heidelberg, Germany) (37).

Enzyme assay and molecular modeling. Recombinant proteins were purified and glycosyltransferase assays were carried out as previously described (7). Kinetic parameters (K_m and k_{cat}) were calculated using a Lin-eweaver-Burk plot. For kinetic parameter determination, enzyme assays were carried out at 37°C for 30 min using 13 μg/ml of enzyme for UDP-glucose and 5 μg/ml for UDP-xylose. Sugar donor (UDP-glucose or UDP-xylose) concentrations varied from 100 to 1,200 μM, and the quercetin concentration was 100 μM. The data represent the averages of three independent measurements ± standard deviations (SD).

The previously constructed comparative model structure of AtUGT78D3 (37) was used as a wild-type enzyme structure, and the AtUGT78D3 H380Q mutant structure was generated through point mutation of His380 to Gln380 in the build module of the molecular modeling software Maestro (Schrödinger). Docking studies of UDP-glucose and UDP-xylose were then conducted using the same protocol as used in our previous study (39). We used the protein preparation utilities in Maestro to assign the charge state of ionizable residues, add hydrogens, and minimize energy. The ligands, UDP-glucose or UDP-xylose, were then docked into the wild-type and mutant model structures using GLIDE. The default settings of the standard precision mode of GLIDE were employed for docking, and up to 10 poses were saved for analysis.

Biotransformation of flavonoids. Overnight cultures of *E. coli* harboring genes of interest in expression vectors were inoculated into 50 ml of fresh LB medium containing appropriate antibiotics and were cultured to an optical density at 600 nm (OD_{600}) of 0.8. Genes were induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Transformants were cultured for an additional 20 h at 18°C, and cells were harvested by centrifugation and resuspended in fresh M9 medium containing 2% glucose, antibiotics, and 1 mM IPTG. To compare the production of quercetin glycosides by the same *E. coli* strain harboring different constructs or by different *E. coli* strains, cell densities of all cultures were adjusted to an OD_{600} of 3. The reactions were performed in 14- by 145-mm test tubes, and the total volume of the reaction mixture was 2 ml. For production of quercetin 3-*O*-xyloside, 100 μM quercetin was added at 0 and 3 h, and the reaction mixture was incubated at 30°C for 8 h. For production of quercetin 3-*O*-arabinoside, 100 μM quercetin was added at 0 h, and the reaction mixture was incubated at 30°C for 3 h.

The mean and the SD were calculated from triplicate experiments. Analysis of variance (ANOVA) was carried out using Tukey's method, with significance at a *P* value of 0.01, using MS Excel (Microsoft Office 2010).

To analyze product formation, cell growth was monitored by determining the absorbance at 600 nm. Culture supernatants were collected, extracted twice with an equal volume of ethyl acetate, and then dried under vacuum. The dried samples were dissolved in dimethyl sulfoxide (DMSO) and analyzed using high-performance liquid chromatography (HPLC). A Varian HPLC equipped with a photodiode array (PDA) detector and a C_{18} reverse-phase column (Agilent; 4.60 by 250 mm; 3.5-μm particle size) was used (40). The structure of the reaction product was determined using nuclear magnetic resonance (NMR) spectroscopy (17). The following data were obtained.

Quercetin 3-O-xyloside. ^{1}H NMR (DMSO-$d6$, 400 MHz): δ 6.20 (d, J = 1.9 Hz, H-6), 6.40 (d, J = 1.9 Hz, H-8), 2.57 (d, J = 2.1 Hz, H-2′), 6.85 (d, J = 8.4 Hz, H-5′), 7.54 (dd, J = 8.4 Hz, 2.1 Hz, H-6′), 5.34 (d, J = 7.3 Hz, H-1″), 3.31 (m, H-2″), 3.19 (m, H-3″), 3.32 (m, H-4″), 2.26 (m, H-5″), and 3.63 (dd, J = 11.4 Hz, 5.1 Hz, H-5″). ^{13}C NMR (DMSO-$d6$, 100 MHz): δ 156.2 (C-2), 133.1 (C-3), 177.3 (C-4), 161.2 (C-5), 98.7 (C-6), 164.3 (C-7), 93.5 (C-8), 156.2 (C-9), 103.8 (C-10), 120.9 (C-1′), 116.0 (C-2′), 144.9 (C-3′), 148.6 (C-4′), 115.3 (C-5′), 121.4 (C-6′), 101.7 (C-1″), 73.5 (C-2″), 76.0 (C-3″), 69.3 (C-4″), and 66.0 (C-5″).

Quercetin 3-O-arabinoside. ^{1}H NMR (DMSO-$d6$, 400 MHz): δ 6.19 (d, J = 1.9 Hz, H-6), 6.40 (d, J = 1.9 Hz, H-8), 7.51 (d, J = 2.2 Hz, H-2′), 6.84 (d, J = 8.5 Hz, H-5′), 7.66 (dd, J = 8.5 Hz, 2.2 Hz, H-6′), 5.27 (d, J = 5.2 Hz, H-1″), 3.75 (m, H-2″), 3.51 (dd, J = 7.1 Hz, 3.0 Hz, H-3″), 3.65 (m, H-4″), 3.21 (dd, J 11.3 Hz, 2.1 Hz, H-5″), and 3.60 (dd, J = 11.3 Hz, 5.3 Hz, H-5″). ^{13}C NMR (DMSO-$d6$, 100 MHz): δ 156.3 (C-2), 133.7 (C-3), 177.5 (C-4), 161.2 (C-5), 98.6 (C-6), 164.2 (C-7), 93.5 (C-8), 156.3 (C-9), 103.8 (C-10), 120.8 (C-1′), 115.7 (C-2′), 144.9 (C-3′), 148.6 (C-4′), 115.3 (C-5′), 122.0 (C-6′), 101.3 (C-1″), 70.7 (C-2″), 71.6 (C-3″), 66.0 (C-4″), and 64.3 (C-5″).

Analysis of UDP-xylose. *E. coli* BL21(DE3) strains harboring *AtUXS* or *AtUXS* and *Ecugd* were cultured in 50 ml of fresh LB medium supplemented with chloramphenicol (50 μg/ml) at 37°C to an OD$_{600}$ of 0.6. IPTG (1 mM) was added to the cultures, and the cells were allowed to grow at 18°C for 24 h. Cells were collected by centrifugation and washed twice with 20 ml of M9 medium containing 2% glucose. Cell density was adjusted to an OD$_{600}$ of 3.0 in 50 ml of M9 medium containing 2% glucose, and the cells were grown at 30°C for 24 h. The cells were collected by centrifugation, washed twice with 50 mM Tris-HCl (pH 7.5), and weighed. Cell pellets were resuspended in 50 mM Tris-HCl (pH 7.5; 50 mg [wet cell weight] per ml of Tris-HCl) and lysed by sonication. Cell debris was removed by centrifugation at 15,000 × g for 15 min, and the supernatants were transferred to Eppendorf tubes. The supernatants were boiled at 100°C for 5 min to denature the proteins, and the denatured proteins were subsequently pelleted by centrifugation at 13,000 rpm for 20 min. The supernatants were analyzed using a Thermo Fisher Ultimate 3000 HPLC system equipped with a C$_{18}$ reverse-phase column (Agilent; 4.60 by 250 mm; 3.5-μm particle size) coupled to a diode array detector (DAD). The reaction products were eluted with buffer A (20 mM *tert*-butylamine-H$_3$PO$_4$ [pH 6.6]) containing 2% (vol/vol) acetonitrile and buffer B (20 mM *tert*-butylamine-H$_3$PO$_4$ [pH 6.6]) containing 20% (vol/vol) acetonitrile. HPLC settings were as follows: 0% B at 0 min, 42.9% B for 15 min, 100% B from 15.5 to 20 min, and 0% B from 20.1 to 25 min. The flow rate was 1 ml/min. The reaction products were monitored by measuring absorbance at 261 nm, which is the maximal wavelength absorbance for UDP-sugars, and the absorption spectra were analyzed over the range of 200 to 500 nm. UDP-xylose was purchased from the Complex Carbohydrate Research Center (University of Georgia, Athens, GA) and was used to generate a standard curve for quantification of the reaction products.

RESULTS

Production of flavonoid O-xylose and flavonoid O-arabinose in wild-type *E. coli*. Most UGTs exhibit sugar donor as well as sugar acceptor specificity, although some UGTs have broad substrate specificity (6, 40). Therefore, biosynthesis of flavonoid O-xyloside and flavonoid O-arabinoside in *E. coli* requires expression of genes for the synthesis of UGTs specific for each nucleotide sugar (UDP-xylose and UDP-arabinose) in the host cell. *E. coli* does not synthesize nucleotide-xylose or nucleotide-arabinose (neither UDP nor TDP derivative [41]). However, it synthesizes UDP-glucuronic acid, which is a substrate for the synthesis of UDP-xylose and UDP-arabinose. To synthesize UDP-xylose and UDP-arabinose in *E. coli*, we engineered the cells to express plant genes encoding UDP-xylose synthase (*UXS*) and UDP-glucose epimerase

TABLE 2 Kinetic parameters of AtUGT78D2 and AtUGT78D3 H380Q for UDP-glucose and UDP-xylose[a]

Enzyme and substrate	K_m (μM^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (nM^{-1} s^{-1})
AtUGT78D2			
UDP-glucose	240.40 ± 10.24	28.12 ± 1.71	116.97 ± 2.04
UDP-xylose	247.06 ± 15.58	0.61 ± 0.01	2.47 ± 0.11
AtUGT78D3 H380Q			
UDP-glucose	108.71 ± 12.78	0.39 ± 0.03	3.59 ± 0.13
UDP-xylose	152.06 ± 12.23	2.26 ± 0.01	14.86 ± 1.04

[a] Enzymatic assays were carried out at 37°C for 20 min using 13 μg/ml of enzyme for UDP-glucose and 5 μg/ml for UDP-xylose. Sugar donor (UDP-glucose or UDP-xylose) concentrations were 100 to 1,200 μM, and the quercetin concentration was 100 μM. K_m and k_{cat} were calculated using Lineweaver-Burk plots. These data represent the averages of three independent measurements ± SD.

(*UGE*) because flavonoid O-xyloside and flavonoid O-arabinoside are commonly found in plants, and biosynthesis of the sugar donors UDP-xylose and UDP-arabinose is well established in plants (10). For synthesis of UDP-xylose, *UXS* was introduced into *E. coli*.

No UGTs have been reported to use UDP-xylose as a sugar donor. However, because glucose and xylose share the same pyranose core structure, UGTs that use UDP-glucose as a sugar donor are likely to also recognize UDP-xylose. Therefore, we tested the ability of AtUGT78D2 to catalyze quercetin 3-O-xyloside synthesis. AtUGT78D2 uses UDP-glucose as a sugar donor. The affinity of purified recombinant AtUGT78D2 for UDP-glucose and UDP-arabinose was examined. Based on k_{cat}/K_m values, the affinity of AtUGT78D2 for UDP-glucose was approximately 47 times higher than that for UDP-xylose (Table 2). In addition, we evaluated the biotransformation of quercetin using strain B400, which harbors AtUGT78D2 and AtUXS. Analysis of the biotransformation product showed that quercetin 3-O-glucose was synthesized as the major product and quercetin 3-O-N-acetylglucosamine as a minor product, as reported previously (42). Only small amounts of quercetin 3-O-xyloside were detected (data not shown). This result was in agreement with enzyme kinetic data.

A different strategy was used to identify a UGT exhibiting higher affinity for UDP-xylose than for UDP-glucose. Molecular docking experiments were conducted with modeled UGTs to find a UGT with high affinity for UDP-xylose. AtUGT78D3, which uses UDP-arabinose as a sugar donor and quercetin as a sugar acceptor, was used for the docking study because its three-dimensional structure had previously been determined (37). UDP-xylose was docked into the substrate-binding site of AtUGT78D3. His380 of AtUGT78D3 was expected to bind to the α-phosphate of UDP-xylose. This binding resulted in a distance of 4.45 Å between the 3-hydroxyl group of quercetin (the sugar attachment site) and the xylose of UDP-xylose (Fig. 1A). This indicated that the binding of UDP-xylose and quercetin to AtUGT78D3 would likely be nonproductive. Analysis of the docking structure showed that substitution of His380 for Glu resulted in stronger binding between UDP-xylose and quercetin due to binding of Glu to the sugar of UDP-xylose (Fig. 1B). In addition, binding of UDP-xylose to AtUGT78D3 H380Q was predicted to be more favorable than binding of UDP-glucose, based on the distance between the 3-hydroxy group of quercetin and the xylose of UDP-xylose (the docking distances for UDP-xylose and UDP-glucose were 3.67 Å and 4.04 Å, respectively) (Fig. 1C). Therefore, the H380Q substi-
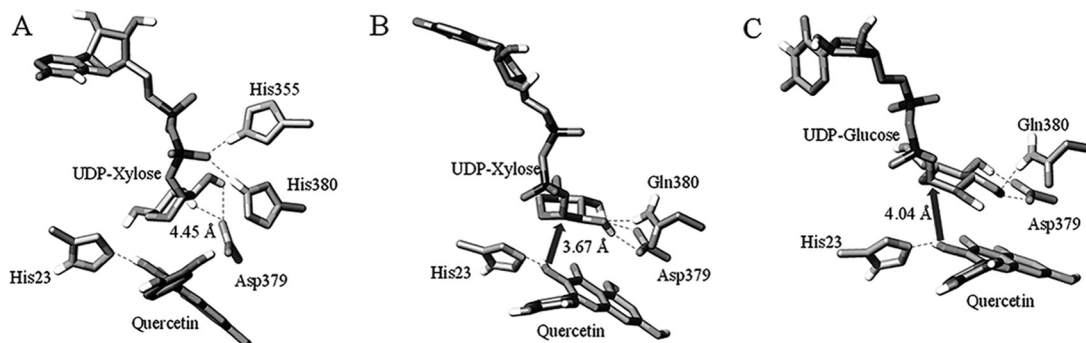
FIG 1 (A) Docking of UDP-xylose into AtUGT78D3. The distance between the 3-hydroxy group of quercetin and the xylose of UDP-xylose is approximately 4.45 Å, and the binding is likely to be nonproductive. (B) Docking of UDP-xylose into AtUGT78D3 H380Q. Gln380 binds to the xylose of UDP-xylose, which moves UDP-xylose closer to the 3-hydroxyl group of quercetin. (C) Docking of UDP-glucose into AtUGT78D3 H380Q. The distance between the 3-hydroxy group of quercetin and the xylose of UDP-xylose is approximately 4.04 Å, which makes UDP-glucose a less favorable sugar donor than UDP-xylose.

tution in AtUGT78D3 was predicted to increase catalytic efficiency for the reaction involving UDP-xylose over that for the reaction involving UDP-glucose. The prediction based on the docking results was tested using site-directed mutagenesis. AtUGT78D3 H380Q exhibited appreciable affinity for UDP-xylose, which was not a substrate of the wild-type AtUGT78D3 (Table 2). Moreover, the affinity of AtUGT78D3 H380Q for UDP-xylose was approximately 4-fold higher than for UDP-glucose, which was expected from the docking analysis. The catalytic efficiency of AtUGT78D3 H380Q for the reaction involving UDP-xylose was approximately 6-fold that of AtUGT78D2.

To test whether the in vitro enzymatic results could be reproduced in vivo, E. coli BL21(DE3) was transformed with both pA-AtUXS and pG-D3Q, and the resulting transformant was named B402 (Table 3). E. coli harboring pA-AtUXS and pG-D3 was named B401. B401 and B402 were then used for biotransformation of quercetin, and the biotransformation products were analyzed using HPLC. Strain B401 did not produce any detectable product, whereas strain B402 produced two detectable products (Fig. 2B). The first peak (P1) in Fig. 2B was for the product with a molecular mass of 464 Da and a retention time indistinguishable from that of quercetin 3-O-glucoside. The molecular mass of the product at P2 in Fig. 2B was 434 Da, indicating that a pentose was attached to quercetin. It was determined to be quercetin 3-O-xyloside by NMR structure analysis (see Materials and Methods). These results showed that expression of AtUXS and AtUGT78D3 H380Q in E. coli resulted in the production of quercetin 3-O-xyloside. However, in these cells, the quantities of quercetin-3-O-glucoside produced were higher than that of quercetin 3-O-xyloside, although the ratio of quercetin-3 O-glucoside to queretin 3-O-xyloside was lower than that in cells expressing AtUGT78D2. It is likely that the levels of UDP-xylose in E. coli were insufficient for synthesis of large quantities of quercetin 3-O-xyloside. Therefore, we engineered the cells to produce higher quantities of UDP-xylose (see below).

For the synthesis of quercetin 3-O-arabinose, UDP-arabinose and a UGT specific for UDP-arabinose and quercetin are required. AtUGT78D3 from A. thaliana is known to transfer arabinose from UDP-arabinose to the 3-hydroxy group of quercetin (43). The construct pG-D3 was generated by subcloning AtUGTD3 into the E. coli expression vector pGEX 5X-2 (Table 3). UDP-arabinose synthesis from UDP-xylose is catalyzed by UXE.

UXE from O. sativa (OsUXE) was subcloned into the E. coli expression vector to obtain pC-OsUXE. For synthesis of UDP-arabinose, pA-AtUXS along with pG-D3 and pC-OsUXE was used to transform E. coli BL21(DE3), and the resulting transformant was named B406 (Table 3). A transformant that did not harbor pC-OsUXE was used as a control. Analysis of the biotransformation products obtained from strain B406 showed a new peak (Fig. 2C) at a retention time different from that of quercetin 3-O-xyloside and a molecular mass of 434 Da (Fig. 2D, bottom graph). NMR results revealed this compound to be quercetin 3-O-arabinoside (see Materials and Methods).

Engineering of E. coli for increased production of flavonoid pentosides. The nucleotide sugar biosynthesis pathways in E. coli are well defined. Engineering of these pathways by gene deletion and/or addition leads to accumulation of specific nucleotide sugars. It was assumed that increasing the production of UDP-glucuronic acid, a precursor for UDP-xylose and UDP-arabinose, would result in increased production of quercetin 3-O-xyloside and quercetin 3-O-arabinoside. Synthesis of UDP-glucuronic acid from UDP-glucose is catalyzed by Ugd. To examine the role of Ugd in the production of UDP-xylose, ugd from E. coli (Ecugd) was expressed in E. coli with AtUXS. As a control, E. coli transformed with only AtUXS was used. The quantities of UDP-xylose produced by the two transformants were compared, and it was found that the strain expressing only AtUXS produced approximately 42.1 μg of UDP-xylose/g (wet weight), whereas the E. coli strain expressing Ecugd and AtUXS produced approximately 161.4 μg of UDP-xylose/g (wet weight). This indicated that overexpression of Ecugd increased UDP-xylose levels 3.83-fold. Next, the effect of elevated UDP-xylose levels on production of quercetin 3-O-xyloside was evaluated. E. coli BL21(DE3) was transformed with pA-AtUXS-Ecugd and pG-D3Q. The resulting E. coli strain, B403 (Table 3), produced 60 mg/liter, approximately 300% more than produced by strain B402, which did not harbor ugd (20 mg/liter [Fig. 3A]). In addition, the ratio of quercetin 3-O-xyloside to quercetin 3-O-glucose in strain B403 was 83:17, whereas in strain B402, it was 3:63 (Fig. 1A).

In E. coli, ArnA (UDP-L-arabinose-4N-formyltransferase/UDP-glucuronic acid C-4″-decarboxylase) converts UDP-glucuronic acid to UDP-4″-ketopentose, which is used for outer membrane lipopolysaccharide synthesis (27). ArnA competes with UXS for UDP-glucuronic acid (Fig. 4). Thus, to increase the avail-

TABLE 3 Plasmids and *Escherichia coli* strains used in this study

Plasmid or *E. coli* strain	Relevant properties or genetic marker	Source or reference
Plasmids		
pACYCDuet	P15A *ori*, Cm^r	Novagen
pCDFDuet	CDF *ori*, Str^r	Novagen
pETDuet	f1 *ori*, Amp^r	Novagen
pGEX 5X-2	pBR322 *ori*, Amp^r	GE Healthcare
pE-AmUGT	pET plus *UGT* from *Antirrhinum majus*	This study
pE-AmUGT-Ecugd	pET plus *UGT* from *A. majus* and *ugd* from *E. coli*	This study
pE-Amugt-Atugd	pET plus *UGT* from *A. majus* and *ugd* from *A. thaliana*	This study
pE-Amugt-Gmugd	pET plus *UGT* from *A. majus* and *ugd* from *Glycine max*	This study
pG-D2	pGEX 5X-2 plus *AUGT78D2* from *A. thaliana*	This study
pG-D3Q	pGEX 5X-2 plus *AUGT78D3H380Q* from *A. thaliana*	This study
pA-AtUXS	pACYCD plus *UXS* from *A. thaliana*	This study
pA-Ecugd	pACYC plus *ugd* from *E. coli*	This study
pA-AtUXS-Ecugd	pACYC plus *UXS* from *A. thaliana* and *ugd* from *E. coli*	This study
pG-D3	pGEX 5X-2 plus *AtUGT78D3* from *A. thaliana*	This study
pC-OsUXE	pCDF plus *UXE* from *Oryza sativa*	This study
Strains		
BL21(DE3)	F$^-$ *ompT hsdS$_B$*(r$_B^-$ m$_B^-$) *gal dcm lon* (DE3)	Novagen
BarnA	BL21(DE3) Δ*arnA*	5
Bpgi	BL21(DE3) Δ*pgi*	42
BarnA-pgi	BL21(DE3) Δ*arn* Δ*pgi*	This study
B400	BL21 harboring pG-D2 and pA-AtUXS	This study
B401	BL21 harboring pG-D3 and pA-AtUXS	This study
B402	BL21 harboring pG-D3Q and pA-AtUXS	This study
B403	BL21 harboring pG-D3Q and pA-AtUXS-Ecugd	This study
B404	BarnA harboring pG-D3Q and pA-AtUXS	This study
B405	BarnA harboring pG-D3Q and pA-AtUXS-Ecugd	This study
B406	BL21 harboring pG-D3, pA-AtUXS-Ecugd, and pC-OsUXE	This study
B407	BarnA harboring pG-D3, pA-AtUXS-Ecugd, and pC-OsUXE	This study
B408	Bpgi harboring pG-D3, pA-AtUXS-Ecugd, and pC-OsUXE	This study
B409	BarnA-pgi harboring pG-D3, pA-AtUXS-Ecugd, and pC-OsUXE	This study

able pool of UDP-glucuronic acid, *arnA* was deleted in *E. coli*, resulting in the strain BarnA (Table 3). The BarnA strain was transformed with either pG-D3Q/pA-AtUXS (strain B404) or pG-D3Q/pA-AtUXS-Ecugd (strain B405). Strain B405 produced 115% (69 mg/liter) more quercetin 3-*O*-xyloside than strain B403 (Fig. 3A). Strain B405 produced 324% more quercetin 3-*O*-xyloside than did strain B402 (Fig. 3A), and the ratio of quercetin 3-*O*-glucoside to quercetin 3-*O*-xyloside shifted from 63:37 to 13:87. Therefore, strain B405 was found to be the best strain for producing quercetin 3-*O*-xyloside.

In *E. coli*, UDP-glucose is a key nucleotide sugar for synthesis of other UDP-sugars. Therefore, increasing the pool of UDP-glucose might increase the supply of substrates for biosynthesis of other UDP-sugars. The reaction catalyzed by Pgi (phosphoglucose isomerase) might be the rate-limiting step in this process. Pgi mediates the interconversion of glucose 6-phosphate and fructose 6-phosphate. Deletion of *pgi* increases the level of glucose 6-phosphate, which is converted to glucose 1-phosphate by Pgm (phosphoglucomutase) and is therefore expected to increase the pool of UDP-glucose. The *E. coli* strain B-pgi, which lacks *pgi*, was transformed with pA-Ecugd-AtUXS and pG-D3Q. However, the strain produced less quercetin 3-*O*-xyloside than the wild type (data not shown).

The production of quercetin 3-*O*-xylose was monitored in strain B405. The optimum cell concentrations were determined.

Cell concentrations over a range of OD$_{600}$s from 1 to 5 were tested in the presence of 100 μM quercetin. Production of quercetin 3-*O*-xylose continued to increase from an OD$_{600}$ of 1 to 3, after which no further increase was observed. Thus, the optimum cell density was at an OD$_{600}$ of 3. In order to monitor the production of quercetin 3-*O*-xyloside, 100 μM quercetin was added at 0, 3, 6, and 9 h to cultures containing the BarnA strain harboring pA-Ecugd-AtUXS and pG-D3Q. Quercetin 3-*O*-xyloside production increased exponentially until 12 h, reaching the maximal level at 30 h. Approximately 150 mg/liter of quercetin 3-*O*-xyloside was obtained (Fig. 5A).

From this result, it was apparent that deletion of *arnA* and expression of *Ecugd* were important factors in the increased production of UDP-arabinose. Therefore, the BarnA strain was transformed with the constructs pA-Ecugd-AtUXS, pC-OsUXE, and pG-D3 to obtain strain B407. Strain B407 produced approximately 114% (23.1 mg/liter) more quercetin 3-*O*-arabinose than strain B406 (20.2 mg/liter) (Fig. 3B). Notably, the *pgi* mutant (strain B408 in Fig. 3B) produced much less (2.1 mg/liter) quercetin 3-*O*-arabinose than the wild-type strain B406.

The cell density at an OD$_{600}$ of 3 was also found to be the optimal density for maximal production of quercetin 3-*O*-arabinoside. Using strain B407, we monitored the production of quercetin 3-*O*-arabinoside in the same manner as for quercetin 3-*O*-
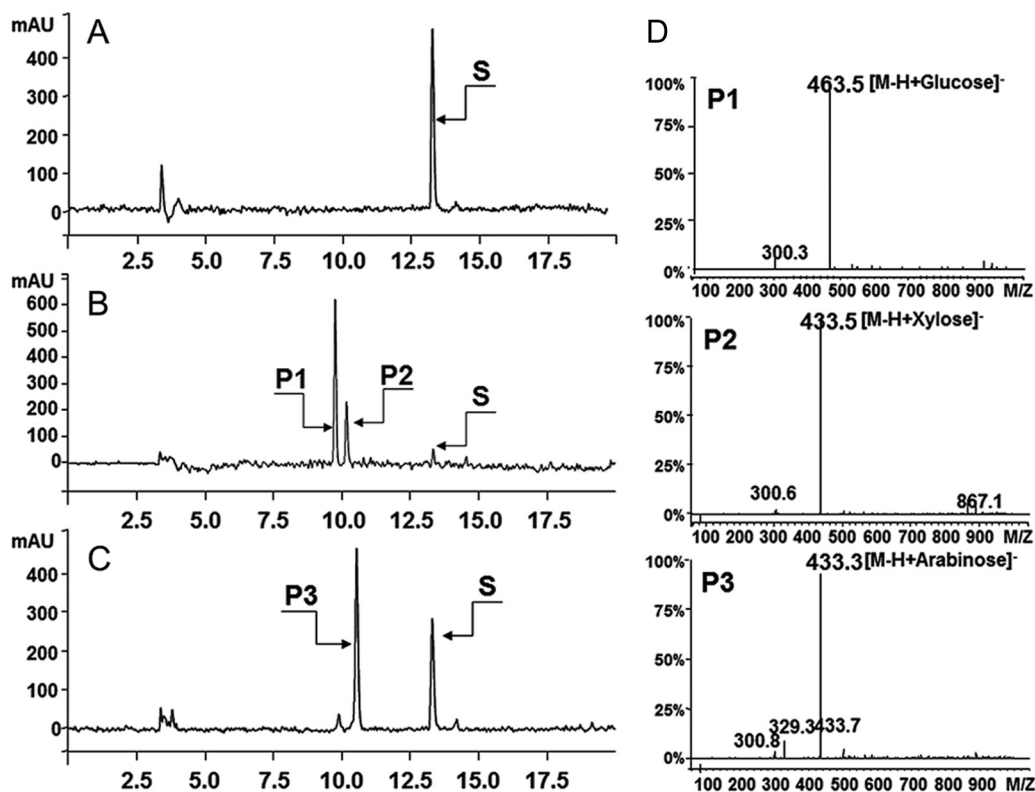
FIG 2 HPLC analysis of biotransformation products. (A) Authentic quercetin (S); (B) biotransformation products obtained from strain B401 (P1 and P2 are biotransformation products); (C) biotransformation products obtained from strain B406; (D) mass spectrometry analysis of the indicated biotransformation products. The mass spectrometry analysis was conducted in negative electrospray ionization mode.

xyloside. Through this approach, 158 mg/liter of quercetin 3-*O*-arabinoside was produced after 84 h of incubation (Fig. 5).

DISCUSSION

E. coli is a good system for the synthesis of flavonoids because it contains transporters to import exogenous flavonoids into the cell and to export the synthesized flavonoids (44). Therefore, analysis of the culture medium is sufficient for monitoring production of flavonoids. In addition, *E. coli* possesses a system for supplying precursors for several cosubstrates such as UDP-sugars and *S*-adenosylmethionine (SAM). In this study, two genes were intro-
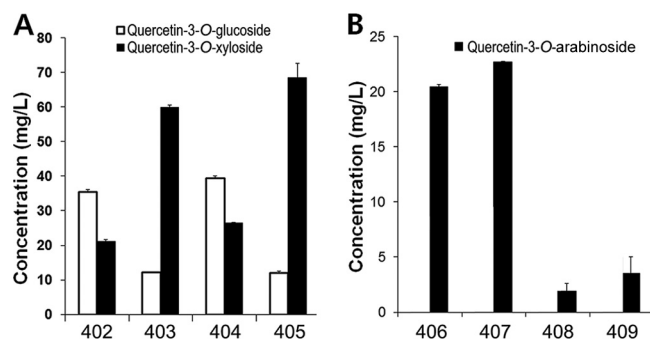

FIG 3 Relative production of quercetin 3-*O*-xyloside and quercetin 3-*O*-glucoside (A) or quercetin 3-*O*-arabinoside (B) in different mutant *E. coli* strains. Three independent experiments were conducted. Error bars represent standard deviations.

duced in *E. coli* to enable synthesis of UDP-xylose and UDP-arabinose using endogenous UDP-glucuronic acid as the precursor. The production of quercetin 3-*O*-xyloside and quercetin 3-*O*-arabinoside by these engineered cells shows that both of these nucleotide sugars were successfully synthesized in the transformants.

Recently, Pandey et al. (19) demonstrated the production of quercetin 3-*O*-xyloside in *E. coli*. This group used an *E. coli* mutant that showed increased UDP-glucose production and expressed a gene for the synthesis of UDP-xylose. Although they optimized the fermentation conditions for the production of quercetin 3-*O*-xyloside, the yield of quercetin 3-*O*-xyloside was approximately 24 mg/liter, which was less than the yield observed in this study. From the findings of previous and current studies, two factors seem crucial for increased synthesis of quercetin 3-*O*-xyloside. First, the amount of substrate limits the yield of the final product, quercetin 3-*O*-xyloside. We chose to increase the level of UDP-glucuronic acid, which serves as a substrate of UXS. We found that the quantities of UDP-glucuronic acid synthesized increased following overexpression of *ugd* and deletion of *arnA*, which, in turn, led to increased quercetin 3-*O*-xyloside production. Pandey et al. (19) increased the pool of UDP-glucose instead of UDP-glucuronic acid by mutating several genes and overexpressing *galU* (encoding glucose 1-phosphate uridyltransferase). Although the pool of UDP-glucose increased, there was likely limited conversion of UDP-glucose into UDP-glucuronic acid. Therefore, the final yield of quercetin 3-*O*-xyloside was limited. The second point to be considered is the selection of glycosyltransferase. We selected and mutated a glycosyltransferase to increase catalytic efficiency for
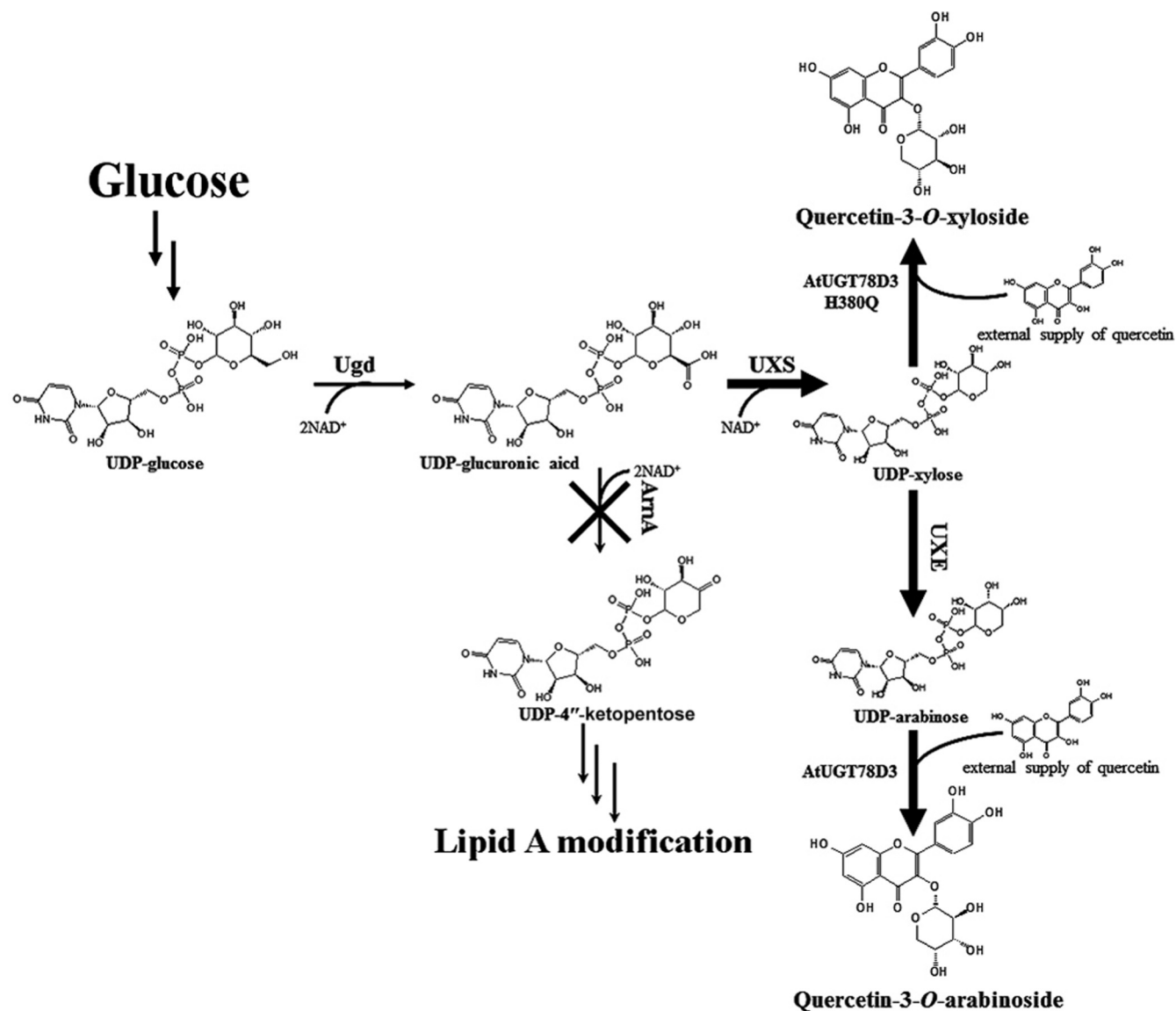
FIG 4 Schematic diagram of synthesis of quercetin 3-O-xyloside and quercetin 3-O-arabinoside. Ugd, UDP-glucose dehydrogenase; ArnA, UDP-ʟ-Ara4N formyltransferase/UDP-GlcA C-4″-decarboxylase; ArnB, UDP-ʟ-Ara4O C-4″ transaminase; AtUXS, UDP-xylose synthase from *Arabidopsis thaliana*; OsUXE, UDP-xylose epimerase from *Oryza sativa*; D3Q, AtUGT78D3 H380Q; D3, AtUGT78D3. The bold black arrows indicate newly introduced biosynthetic genes in *E. coli* for quercetin 3-O-pentoside production.

UDP-xylose at the expense of UDP-glucose efficiency. These strategies together enabled increased production of quercetin 3-O-xyloside.

Strain B-pgi, which was transformed with pA-Ecugd-AtUXS and pG-D3Q, produced less quercetin 3-O-xyloside than the wild type (data not shown). Deletion of *pgi* redirects energy metabolism and likely inhibits cell growth (45). Therefore, reduced growth of B-pgi could be the reason for the mutant producing less quercetin 3-O-xyloside than the wild type.

There are two possible explanations for strain B400 producing only quercetin 3-O-glucoside. First, AtUGT78D2 likely has much higher affinity for UDP-glucose than for UDP-xylose because it is a flavonol 3-O-glucosyltransferase (46), and kinetic analysis showed that it exhibited greater catalytic efficiency for UDP-glucose than for UDP-xylose. Second, UDP-xylose levels might be

much lower than UDP-glucose levels in B400. Therefore, supplementation of the sugar donor was not sufficient for the production of quercetin 3-O-xyloside. In addition, results from our previous study, wherein we demonstrated quercetin 3-O-N-acetylglucosamine synthesis in *E. coli* expressing AtUGT78D2 (17), showed that the ratio of the sugar donors (UDP-glucose and UDP-N-acetylglucosamine) was highly important. AtUGT78D2 showed considerably higher affinity for UDP-glucose than for UDP-N-acetylglucosamine *in vitro*. However, the limited availability of UDP-glucose *in vivo* could shift the affinity from UDP-glucose to UDP-N-acetylglucosamine (17). Therefore, instead of testing the second possibility, we tried to identify a UGT with higher affinity for UDP-xylose than for UDP-glucose by conducting molecular docking experiments with modeled UGTs to find the UGT exhibiting affinity for UDP-xylose.
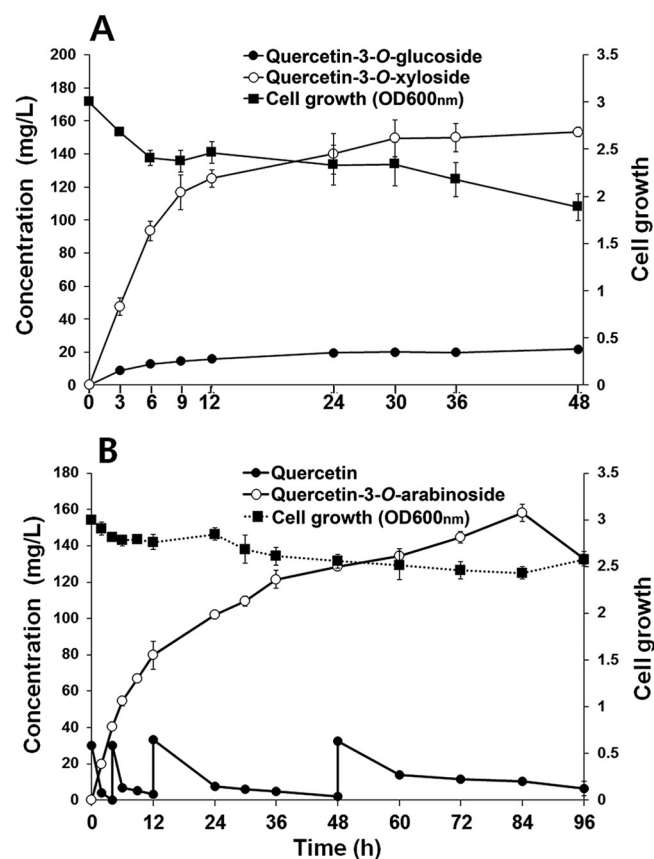
FIG 5 Production of quercetin 3-*O*-xyloside (A) and quercetin 3-*O*-arbinoside (B) by biotransformation of *E. coli* strain B405 and strain B407, respectively. Initially 100 µM quercetin was added, and then an additional 50 µM was added at 6, 12, and 48 h. Therefore, the final concentration of quercetin was 250 µM (75.5 mg/liter). Three independent experiments were conducted. Error bars represent standard deviations.

Plant UGTs have a consensus sequence at the C terminus called the PSPG motif (3, 47). This motif is known to be critical for the recognition of the sugar donor. One of the amino acids in this motif (Glu at 381 of AtUGT78D2) was shown to be involved in sugar donor selectivity (48) and is highly conserved in many plant UGTs (47). Two other amino acids (aspartic acid and histidine) have been found to be involved in sugar donor selectivity (47). Substitution of Glu for His at 381 of AtUGT78D2 resulted in a failure to recognize UDP-glucose as well as UDP-xylose. Therefore, we searched for a new UGT that had a higher catalytic efficiency for UDP-xylose than for UDP-glucose. Molecular docking with the modeled UGT structure was used because it is a rapid virtual screening approach. We focused on the highly conserved UDP-sugar recognition amino acid residue Glu380. AtUGT78D3, which was the template for the mutation, specifically uses UDP-arabinose as a sugar donor and contains a His as the conserved UDP-sugar recognition amino acid residue. Replacement of His 380 with Gln in AtUGT78D3 conferred on the enzyme the ability to recognize UDP-xylose and UDP-glucose and resulted in the loss of the ability to recognize UDP-arabinose.

In conclusion, approximately 160 mg/liter of both quercetin 3-*O*-xyloside and quercetin 3-*O*-arabinoside was successfully synthesized in *E. coli* through metabolic engineering of the nucleotide

biosynthetic pathway and protein engineering of the glycosyltransferase. This approach will be useful for obtaining sufficient quantities of quercetin 3-*O*-pentosides for studying their diverse biological activities. In addition, the *E. coli* strains that were engineered to produce UDP-xylose and UDP-arabinose can be used to glycosylate other small molecules by expressing a suitable UGT.

ACKNOWLEDGMENTS

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